Exhibit 5

ELSEVIER

Journal of Controlled Release 83 (2002) 401–414

journal of controlled release

www.elsevier.com/locate/jconrel

Study of the drug release mechanism from tyrphostin AG-1295-loaded nanospheres by in situ and external sink methods

Michael Chorny M.Sc.[a], Ilia Fishbein M.D. Ph.D.[a], Haim D. Danenberg M.D.[b], Gershon Golomb Ph.D.[a,*]

[a]*Department of Pharmaceutics, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, POB 12065, Jerusalem 91120, Israel*

[b]*Department of Cardiology, Hadassah-Hebrew University Hospital, Jerusalem, Israel*

Received 17 May 2002; accepted 1 August 2002

Abstract

The present study focused on in vitro release of polylactide-nanoencapsulated tyrphostin AG-1295, a potential agent for local therapy of restenosis. The drug was formulated in matrix-type nanoparticles, termed nanospheres (NS) using the nanoprecipitation method. AG-1295 is a model for low-molecular weight lipophilic compounds, the release behavior of which cannot be adequately characterized by existing methods. An in vitro release technique suitable for optimizing the nanoparticulate formulation release behavior was developed through a novel external sink method and an in situ release method utilizing the environmental sensitivity of the AG-1295 fluorescence spectrum. Similar tendencies were demonstrated by both methods in drug release studied as a function of selected NS preparation variables. The release properties of the drug fractions varying in their binding mode to the carrier particles were studied by the external sink method. The NS surface-adsorbed drug exhibited a significantly higher release rate compared to the drug entrapped in the polymeric matrix. The in situ release of the encapsulated drug was analyzed using the diffusion models of release from a matrix-type sphere. The release was shown to be a composite process, with a burst phase attributed largely to the rapid dissociation of the surface-bound AG-1295. The diffusion-controlled phase exhibited an alteration in kinetic pattern obviously due to the drug distribution between polymeric matrix compartments differing in their permeability. Drug in vitro release investigation may be effectively used to characterize the drug–carrier interaction and internal carrier structure in nanoparticulate formulations, as well as optimize the release behavior in respect to their therapeutic application.

Keywords: Nanospheres; Nanoparticles; Nanoprecipitation; Tyrphostin; Drug release; Controlled release; Diffusion

1. Introduction

Tyrphostin AG-1295, a potent PDGF-receptor β blocker, has been shown to have a significant therapeutic effect in rat and pig injury models of restenosis [1,2]. The drug was encapsulated in polylactide-based nanospheres (NS) using a modified nanoprecipitation method, and the nanoparticulate formulation was subsequently characterized in vitro [3] and in vivo [4]. The local drug residence profile

*Corresponding author. Tel.: +972-2-675-7504; fax: +972-2-675-7503.

E-mail address: golomb@cc.huji.ac.il (G. Golomb).

0168-3659/02/$ – see front matter
PII: S0168-3659(02)00210-9

in the rat arteries correlated well with the antirestenotic effect of the formulation. The in vivo efficacy, as well as drug disposition and elimination kinetics, are affected significantly by formulation properties, such as NS size and drug release kinetics [4]. While 90-nm NS showed a higher tissue uptake compared to 160-nm NS, the delivery of the former also resulted in a faster drug elimination rate from the injury site. This suggests that an optimized drug carrier should ideally combine the better ingress properties achieved by a smaller size with a slower drug release rate from the NS. The tools provided by the nanoprecipitation method for optimizing the formulation in respect to these characteristics are discussed elsewhere [3]. While a comparative study of the formulation variables affecting the NS size is a relatively simple task due to a number of well-established and reliable methods [5], no clear agreement exists on the suitability of the techniques reported to date for drug release determination from colloidal particles in the nanometer size range. The in vivo examination of drug released directly in the target tissue, though usually difficult to conduct, is considered to be of ultimate value in most cases. The difficulty of performing in vivo release studies, as well as the technical limitations of the in vitro methods developed so far, are the main reason for the continuous development of new in vitro drug release techniques and modifications of the known methods [6–10].

In practice, it is difficult to extract valuable information from the release data if the studied formulation is not diluted sufficiently (non-sink conditions) [11]. Sink conditions are reasonably satisfied when the drug concentration in the release medium is kept below 10% of the drug solubility, while the lower applicable limit of the drug concentration is stated by the sensitivity of the analytical method used for determination of the drug. Sink condition is hard to achieve using an aqueous medium with poorly water-soluble drugs, therefore it is difficult to provide a satisfactory design for their release studies. In such case, utilization of a sink medium which is a good solvent to the studied drug and an absolute non-solvent to the particle-forming polymer, may alternatively be considered.

In most techniques the discrimination of the released and carrier-bound drug is accomplished by a physical separation of the colloidal drug carrier from the release medium. The results obtained using these methods reflect complex kinetics of several simultaneous processes, and therefore their interpretation may be highly misleading [10,12]. In a limited number of studies an experimental design avoiding the physical separation and associated distortions in the observed release profile was applied [7,13–15]. This was possible due to the unique properties of the nanoencapsulated compounds utilized for spectroscopic discrimination of the free and bound substance [16]. Yet the spectral properties essential for successful application of such a technique, termed in situ release method, are rarely exhibited by medicinal substances, limiting its application mainly to specially designed model compounds.

In this study the release properties of biodegradable polymeric NS loaded with the antirestenotic agent, tyrphostin AG-1295 [1,3,4,17], have been examined by two methods: an in situ release method based on environment-sensitive fluorescence of AG-1295, and an external sink method utilizing heptane as a release medium to address the strongly lipophilic nature of the active compound. The release kinetics determined using both methods in selected NS formulations are presented in comparison. The specific advantages of each method have further been exploited to examine the release properties of the drug encapsulated versus adsorbed to the NS surface, and a possible mechanism of the drug release is proposed.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide), M_w 90 000–120 000 g/mol and Pluronic F-68 (Poloxamer 188 NF) were purchased from Sigma (St. Louis, MO, USA). AG-1295 (6,7-dimethyl-2-phenylquinoxaline) was purchased from Oz Chemicals (Jerusalem, Israel). All organic solvents were of analytical and HPLC grade.

2.2. NS formulation

The nanospheres were prepared using a modified nanoprecipitation method as described elsewhere [3].

A reference formulation was prepared as follows: 200 mg of PLA and 3.0 mg AG-1295 were dissolved in an organic mixture consisting of 19.5 ml acetone and 0.5 ml dichloromethane. This organic solution was poured under moderate stirring on a magnetic plate into an aqueous phase prepared by dissolving 50 mg Pluronic F-68 in 40 ml bidistilled water. The organic solvents were evaporated under atmospheric pressure at room temperature. The volume of the resulting dispersion was adjusted to 10 ml under 12 mm Hg at 35 °C using rotary evaporation (Rotavapor R-124, Büchi, Switzerland). The aggregates were removed by filtration through 0.45-μm hydrophilic syringe filter (Sartorius, Germany). The following variables were chosen for examining the effect of preparation protocol modifications: (a) polymer formulation amount (PLA: 100, 200, 300 mg), (b) polymer non-solvent formulation amount (ethanol: 0, 1, 2 ml), (c) organic solvent elimination rate (atmospheric pressure at room temperature, pressure reduced gradually from 180 to 40 mmHg at 35 °C, pressure reduced instantaneously to 40 mmHg at 35 °C).

2.3. NS size and morphology

NS morphology was observed by means of transmission electron microscopy (CM12, Philips, Holland). NS size was determined by photon correlation spectroscopy (Sub-micronial particle analyzer, Coulter Electronics, USA) in NS suspension diluted with bidistilled water at 25 °C.

2.4. NS drug content

An aliquot of NS aqueous suspension was evaporated to dryness by rotoevaporation under reduced pressure at 35 °C. The residue was dissolved in dichloromethane, and the drug was quantified spectrophotometrically at 265 nm.

2.5. Drug release comparative study

The in situ drug release was performed once for each formulation; the external sink release experiments were performed in duplicate. The error bars represent the standard deviation of the release duplicates at each time-point.

2.5.1. External sink release method

Two 0.5-ml aliquots of freshly prepared NS suspension were placed in test tubes, mixed with accurately weighed 3.0 ml amount of heptane (Sigma, St. Louis, MO, USA) and placed on a Labquake shaker (Labindustries, CA USA) at 25 °C. The upper organic layer was withdrawn completely and replaced with fresh heptane at given time-points. The amount of released drug was determined spectrophotometrically at 262 nm.

2.5.2. In situ release method

Pluronic F-68 was dissolved in bidistilled water at room temperature to yield 6% aqueous solution (acceptor phase). A 50-μl aliquot of freshly prepared NS suspension was mixed with 50 ml of the acceptor phase and placed on stirring on a magnetic plate at 25 °C. The emission spectrum of the samples withdrawn at predetermined time intervals was measured using spectrofluorimeter (Jasco, Japan) without prior separation of the NS from the release medium (λ_{exc}=350 nm).

Calibration was performed by plotting the fluorescence intensity at 400 nm (AG-1295 emission maximum) against drug concentration for (a) AG-1295 solutions in acceptor phase incubated for 24 h with blank NS, (b) NS prepared with increasing formulation amounts of AG-1295, diluted 1:1000 with acceptor phase. The spectrum obtained from blank NS was used to correct for non-specific signal.

The amount of AG-1295 released from the NS was calculated using the following equation:

$$\Phi_t = (C_F)_t \phi_F + (C_B)_t \phi_B \quad (1)$$

where Φ_t is the total AG-1295-related fluorescence measured at time t; ϕ_F and ϕ_B are the specific fluorescence coefficients derived from the calibration plot for the free and bound drug, respectively; $(C_F)_t$ and $(C_B)_t$ are concentrations of the free and bound drug at time t, respectively. $(C_F)_t$ and $(C_B)_t$ are related by the mass balance equation:

$$C = (C_F)_t + (C_B)_t \quad (2)$$

where C is the total drug concentration calculated from NS drug content determined spectrophotometrically as described above.

2.6. Surface adsorbed drug release simulation

A reference formulation was prepared and its drug content was determined as described above. Six portions 1.0 ml each were sealed in dialysis membrane bags with a cut-off of 14 000 kDa (Sigma). The bags were placed in a beaker and the NS were continuously washed with water that was replaced at a rate of 2 l/h using a water circulation system. The drug content change in the washed NS was monitored for 4 h to establish the end of the fast phase of drug removal attributed to loosely bound AG-1295. The amount of drug washed from the NS was obtained from the difference in the drug content before and after the washing procedure. The external sink method was used to determine drug release behavior of the washed and unwashed NS. The release behavior of the surface-adsorbed drug fraction in the reference formulation was determined as follows:

$$\frac{M_{t,\text{ads}}}{M_{\infty,\text{ads}}} = \frac{(M_{t,\text{unwashed NS}} - M_{t,\text{washed NS}})}{d} \quad (3)$$

where $M_{t,\text{ads}}$ is the amount of drug desorbed from NS surface at time t; $M_{\infty,\text{ads}}$ is the total amount of surface adsorbed drug; $M_{t,\text{unwashed NS}}$ and $M_{t,\text{washed NS}}$ are absolute amounts of drug released at time t from the unwashed and washed NS, respectively; d is the difference in the drug content between the unwashed and washed NS.

The deconvoluted release pattern of the washable drug fraction was confirmed by comparison with actual release rate of AG-1295 adsorbed to the surface of blank NS.

2.7. The effect of NS size on drug adsorption and release behavior

The effect of surface area on the amount of drug adsorbed was studied using NS of different size. Two formulations of blank NS were prepared with and without PLA non-solvent (ethanol) in organic phase to produce smaller and larger sized NS, respectively [3]. The obtained NS were filtered using 0.45-μm filter as described above to remove aggregates. Aliquots of the blank NS suspensions were evaporated to dryness for polymer amount determination. Nine ml of each formulation were stirred for 5.5 h with 10 mg of powdered AG-1295 (63-μm aperture sieve). The NS were separated from the free drug by filtration and the drug content was determined. The release behavior of the surface-adsorbed drug was examined by the external sink method. The distortion of the results due to the possible drug solubilizing by the surfactant and/or the residual solvents was assessed in control experiments where AG-1295 was added to a solution obtained at the end of the blank NS preparation procedure without polymer.

2.8. AG-1295 solubility in polymer

AG-1295 solubility in the polymer was determined from drug partitioning between PLA and PLA non-solvent liquid phase. Three mg of accurately weighed AG-1295 were dissolved with PLA (450 or 600 mg) in acetone to yield 3 ml solution. A 1-ml aliquot of the solution was further diluted 1:10 with ethanol (PLA non-solvent) to precipitate the polymer. The liquid phase was clarified using a 1.0-μm glass-fiber prefilter (Millipore, Bedford, MA, USA). A solution obtained similarly by dilution with acetone was used as a reference to calculate the drug concentration in the polymer. The drug amount in the filtrates was determined spectrophotometrically after evaporation to dryness and dissolving the residue in dichloromethane as described above for NS drug content determination.

The acetone extraction from the PLA precipitate upon dilution with ethanol was confirmed by a control experiment performed as described above with no drug added. The acetone concentration in the liquid phase clarified with 1.0-μm glass-fiber prefilter was determined spectrophotometrically at 272 nm.

2.9. Kinetic model of drug release

The NS were prepared as described above, and drug release in situ was performed in duplicate. The obtained data was analyzed for best fit approximation by model equations using Solver algorithm of Microsoft Excel 98 software.

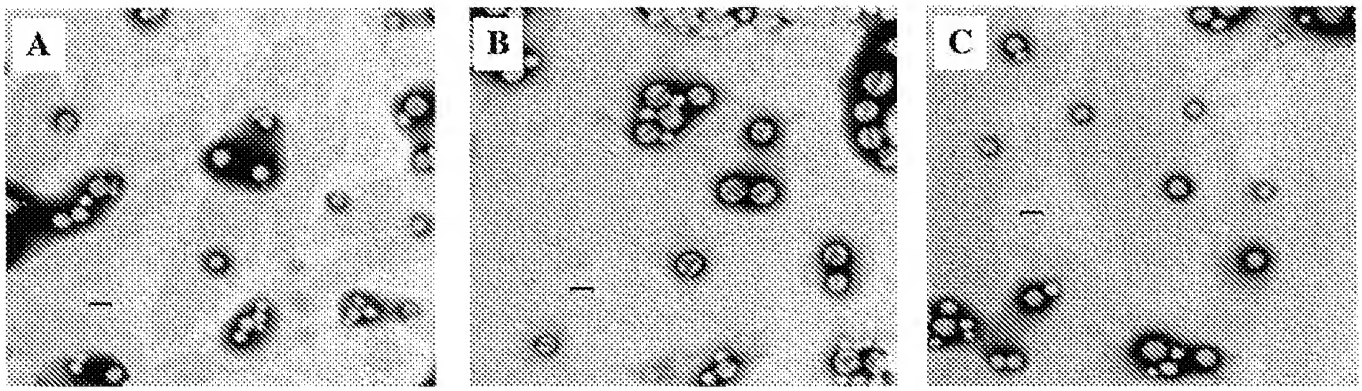

Fig. 1. Transmission electron microscopy images of AG-1295-loaded nanospheres (average size 115 nm, loading 1.5%, w/w) at different time-points of drug release in heptane (external sink method): prior to release (A); 4 h (B); 20 h (C). The released drug fraction was 50 and 95% after 4 and 20 h, respectively. The bar represents 100 nm.

3. Results

The AG-1295-loaded NS were spherical in shape (Fig. 1). NS samples examined following 4 and 20 h of drug release in heptane (external sink method) showed no morphological changes compared to the NS observed prior to the release experiment (Fig. 1A–C).

The in situ release method applied in this study is based on the difference in fluorescence intensity between encapsulated and free AG-1295. The ratio of the specific fluorescence intensities between free and NS-bound drug derived from the slopes of the respective calibration plots (Fig. 2) was 4.2–4.5. A small reduction in emission intensity of the free AG-1295 occurred after incubation for 24 h with blank NS apparently due to partial association of the drug with the NS. However, no further changes in the fluorescence pattern were evident after an additional 48 h of incubation (data not shown) indicating steady state.

The release rate determined by external sink and in situ release methods was considerably reduced by higher PLA formulation content (Fig. 3A,B), while the incorporation of PLA non-solvent and the more intense organic solvents' evaporation were associated with more rapid drug release (Fig. 3C–F). In general, the drug release kinetics determined using both release techniques was similarly affected by the examined formulation variables. However none of the formulations completed the release in situ, while 24–48 h were sufficient to achieve 100% release using the external sink method (data not shown). The initial drug release rate determined in situ was considerably higher than that obtained by the external sink method.

The latter method was utilized to characterize the release behavior of NS surface adsorbed drug. The steady state of drug adsorption on smaller and larger sized blank NS utilized in this experiment (70 ± 21 and 161 ± 19 nm, respectively) was reached after 5.5 h of incubation with powdered AG-1295 (Fig. 4A). The steady state ratio of the drug amount associated with smaller and larger NS, calculated per mg dry weight, was 2.02. AG-1295 was consistently undetectable in the control solution. The release behavior of the drug adsorbed by NS of both sizes was similar (Fig. 4B).

The actual release of the surface adsorbed AG-1295 was further compared to a deconvoluted release behavior of the washable drug fraction in the reference formulation. The release of the loosely bound drug, which accounted for 8.5% of the total drug loading, showed fair correlation with the adsorbed drug release profiles (Fig. 4B), but was considerably different from that of the encapsulated drug (Fig. 4C). The estimated release $t_{50\%}$ was 0.7 h and 4.5 h and the amount released within 7 h was 100 and 63% for the loosely bound and encapsulated drug, respectively.

The drug solubility in the polymer was determined from its partitioning between the solid PLA and organic liquid phases assuming distribution proportional to the ratio of the drug solubilities in the

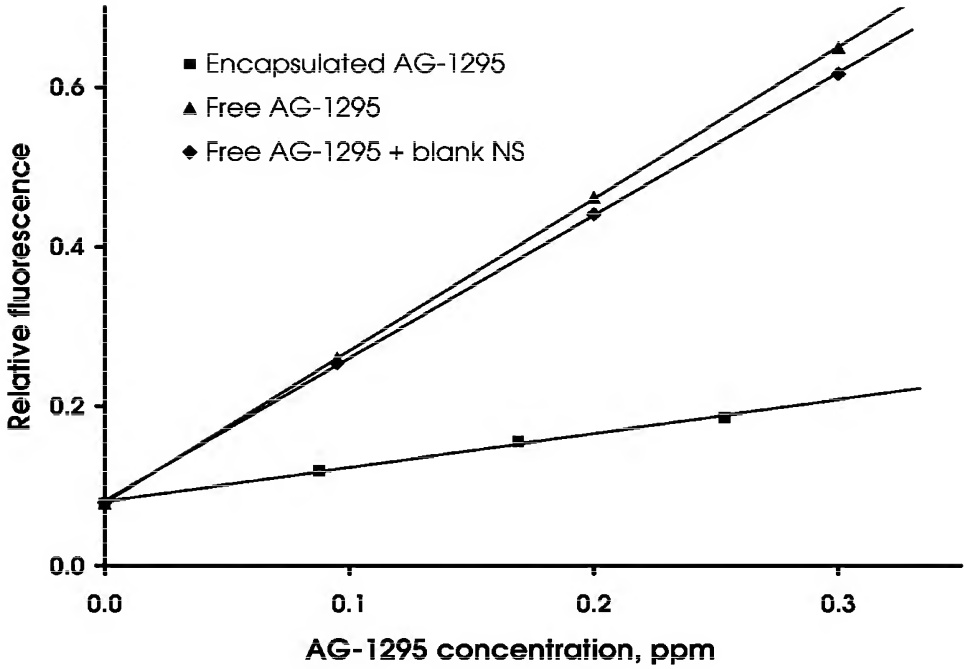

Fig. 2. In situ drug release calibration plot (medium consisting of Pluronic F-68 6% aqueous solution: emission intensity of AG-1295 (λ_{exc}=350 nm, λ_{em}=400 nm) as a function of drug amount (encapsulated versus free). The fluorescence intensity of free drug in the presence of blank nanospheres remained practically unchanged after incubation for 24–72 h. The encapsulated to free drug signal ratio was measured on several occasions and equaled 0.208–0.232.

respective phases. In the two experiments 12.2 and 14.3% of the total amount of AG-1295 (1.0 mg) associated with 150 mg and 200 mg of the precipitated polylactide, respectively, corresponding to 0.76±0.05 μg AG-1295 per mg PLA. It was verified in a separate experiment that the acetone used to dissolve the polymer and the drug was completely extracted by ethanol. The AG-1295 solubility in PLA, calculated using the obtained partition ratio and previously determined drug solubility in 8.7% solution of acetone in ethanol (8.86 mg/ml), was 75.0±4.9 mg/g.

Fig. 5 shows the experimental data of in situ release from (170±20) nm NS fitted by the early-time approximation (4) and late-time approximation (5) of a diffusion-based drug release model from a spherical monolithic carrier [18,19]:

$$\frac{M_t}{M_\infty} = 6\left(\frac{Dt}{r^2\pi}\right)^{1/2} - \frac{3Dt}{r^2} \tag{4}$$

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2}\exp\left(\frac{-\pi^2 Dt}{r^2}\right) \tag{5}$$

where M_t is the amount of the drug which diffuses out of the sphere in time t, M_∞ is the total amount of the drug, D is the diffusion coefficient, r is the radius of the sphere.

The early- and late-time approximation were applied to the drug release within 0–10 h (released drug fraction <60%), and 6.5–72 h (released drug fraction >50%), respectively. The corresponding diffusion coefficients were $(4.14\pm1.09)\times10^{-16}$ and $(1.95\pm0.45)\times10^{-16}$ cm^2/s, respectively.

The release data were also analyzed by the semi-empirical model of diffusional release by Ritger and Peppas [20,21]:

$$\frac{M_t}{M_\infty} = kt^n \tag{6}$$

where k is a correlation constant and n is the diffusional exponent. The diffusional exponent was obtained from the best fitting curve provided by the model and from the slope of log (M_t/M_∞) plotted against log t (Fig. 6), as suggested by Sinclair and Peppas [22], and equaled 0.46±0.03.

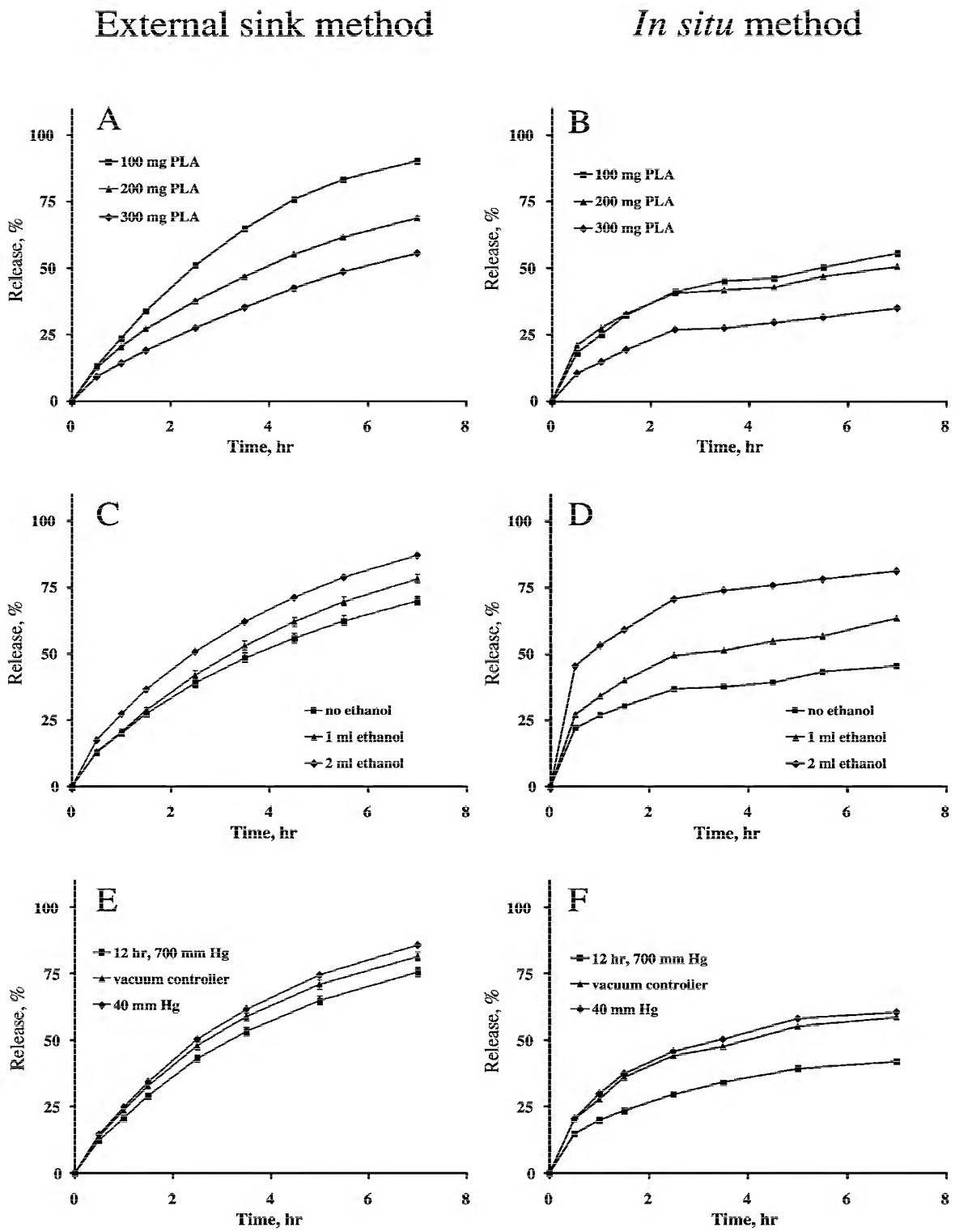

Fig. 3. The release profile of AG-1295 from nanospheres as function of formulation variables obtained by the external sink (left column) and in situ (right column) methods, respectively: PLA amount (A, B); ethanol amount in organic phase (C, D); organic solvents evaporation rate (E, F).

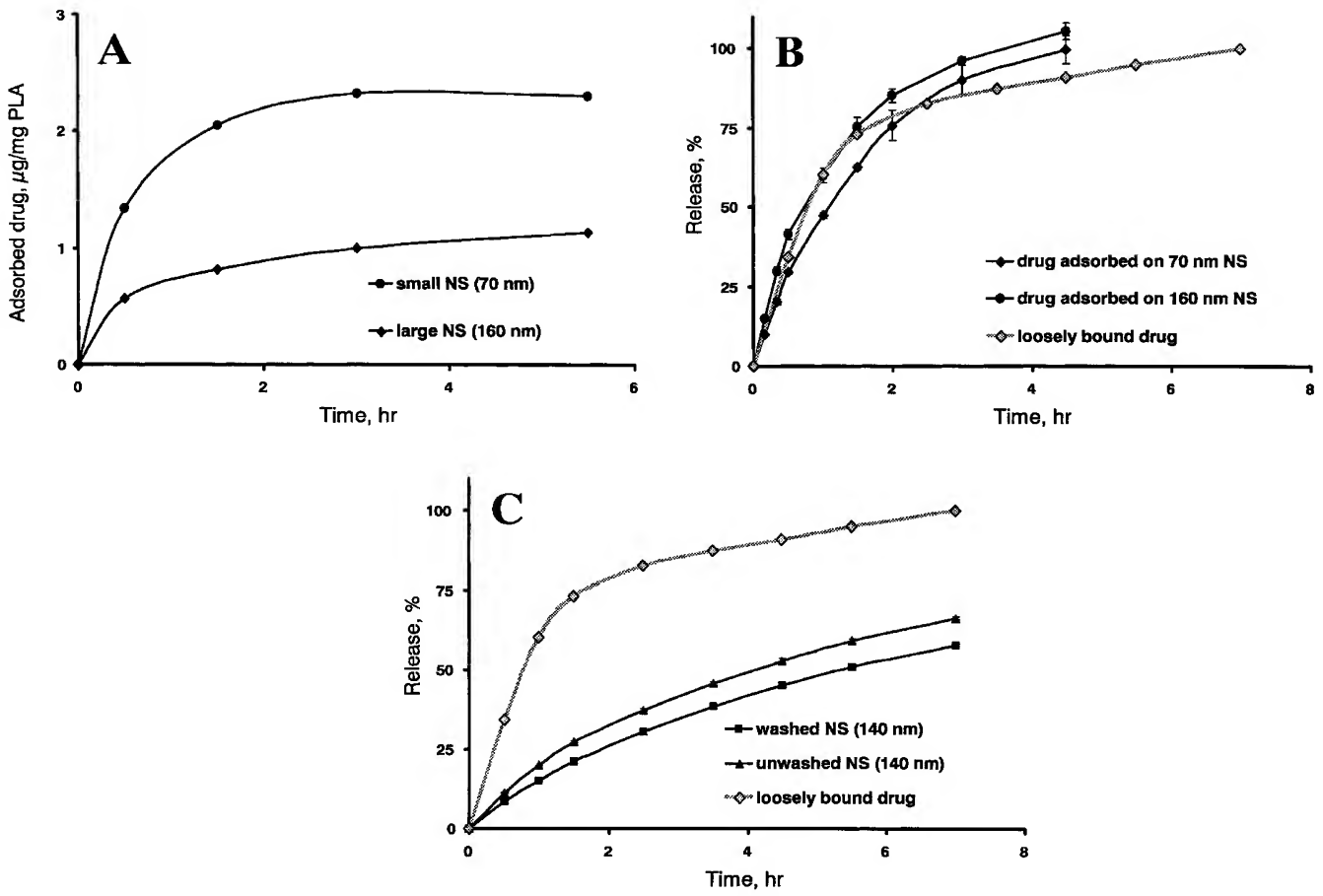

Fig. 4. The kinetics of AG-1295 adsorption on blank nanospheres (A) and the release profile of surface adsorbed drug. The amount of drug adsorbed on 160- and 70-nm blank nanospheres was 19 and 45.6 μg/ml corresponding to 1.13 and 2.29 μg per mg PLA, respectively. The release of AG-1295 adsorbed on the surface of 160- and 70-nm sized blank nanospheres (B) is presented in comparison with the release profile of the loosely bound drug fraction in the reference formulation. The latter was obtained by comparing the release profiles of AG-1295-loaded nanospheres (140 nm) before and after washing with water (C) (see text for details). The drug release from the nanospheres with and without washing is presented as percent of the drug amount in the original formulation (215 μg/ml).

4. Discussion

Depending on their design, in vitro release studies may be used for several purposes: simulation of the drug release behavior in vivo, optimization of the release properties based on comparison between formulations, and investigation of the drug release mechanism which often provides important information about the internal structure of the carrier and the drug–carrier physicochemical relationship. An appropriately designed in vitro release study is often difficult to conduct because of a number of technical problems associated with it [23]. Sink conditions are rarely achievable for a lipophilic drug during the whole course of a release experiment, since the sensitivity of the analytical assay usually does not allow for sufficient carrier dilution in commonly used aqueous acceptor media. Another problem inherent to all release methods where the free drug is not determined in situ, is possible masking of the actual release profile due to the physical separation of the carrier and released drug [10,12]. Because of

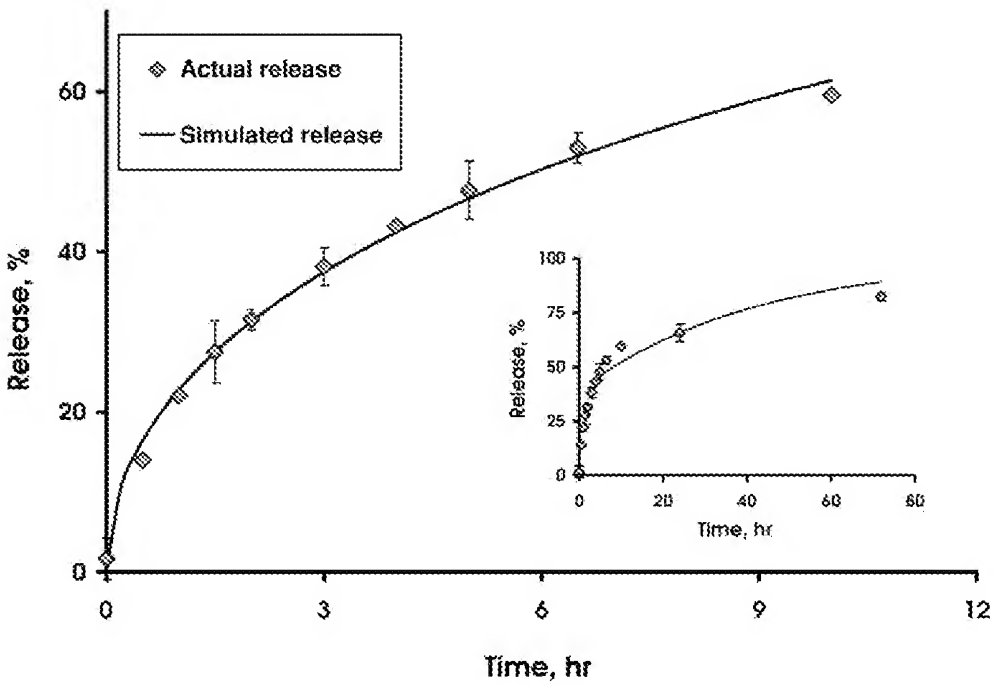

Fig. 5. The in situ release data of AG-1295-loaded nanospheres (170 nm) fitted by the early- and late-time approximation (insert) of the ab initio diffusional release model of Guy et al. [18] (see Eqs. (4) and (5) in the text). The drug release profile exhibited a considerable change in its pattern during the late-time phase (10–72 h) as manifested by a lower released drug fraction values in comparison to the values predicted by the simulation assuming no change in the diffusion coefficient (82 vs. 100% fractional release after 72 h, respectively).

these interferences the examination of the early-time drug release is often inaccurate, while in extreme cases the data obtained during the whole course of an experiment may be of little relevance [10]. A number of improvements in release methods addressing the results' error associated with the separation have been successively reported [8,9,24], none of which, however, was capable of providing a satisfactory solution for the early-time release distortion. Indeed the suitability of a method has to be critically evaluated for each particular application.

With these limitations in mind, we focused on the development and evaluation of a suitable in vitro release technique for PLA nanoencapsulated AG-1295, a highly lipophilic low-molecular weight tyrphostin compound intended for local therapy of restenosis [1,2,25]. In the in situ release method developed for the purposes of this study the NS are diluted directly into the sink medium. The released drug was assayed in the sink utilizing environmentally sensitive fluorescence of AG-1295. The release profile produced by this technique is therefore not affected by the NS separation from the assayed medium [7]. Sink conditions during the whole course of the release experiment were achieved by diluting the studied NS sample 1:1000 with 6% aqueous solution of Pluronic F-68. To our knowledge in situ release studies of medicinal substances have not been reported with one exception, where a method similar to the one presented here had been applied for poly(butyl-2-cyanoacrylate) nanoparticles of adriamycin [13].

The external sink method presented in this study employs continuous extraction of the released AG-1295 by heptane forming a liquid phase immiscible with the aqueous suspension of the carrier NS. Sink conditions are maintained during the experiment due to the good drug solubility in heptane (3 g/l). Heptane was chosen as an acceptor phase since it is a non-solvent to PLA and has no effect on NS morphology. It is also chemically inert thereby providing excellent stability of the drug solutions, almost odorless, considered to be a solvent with low toxic potential, and has limited volatility at room

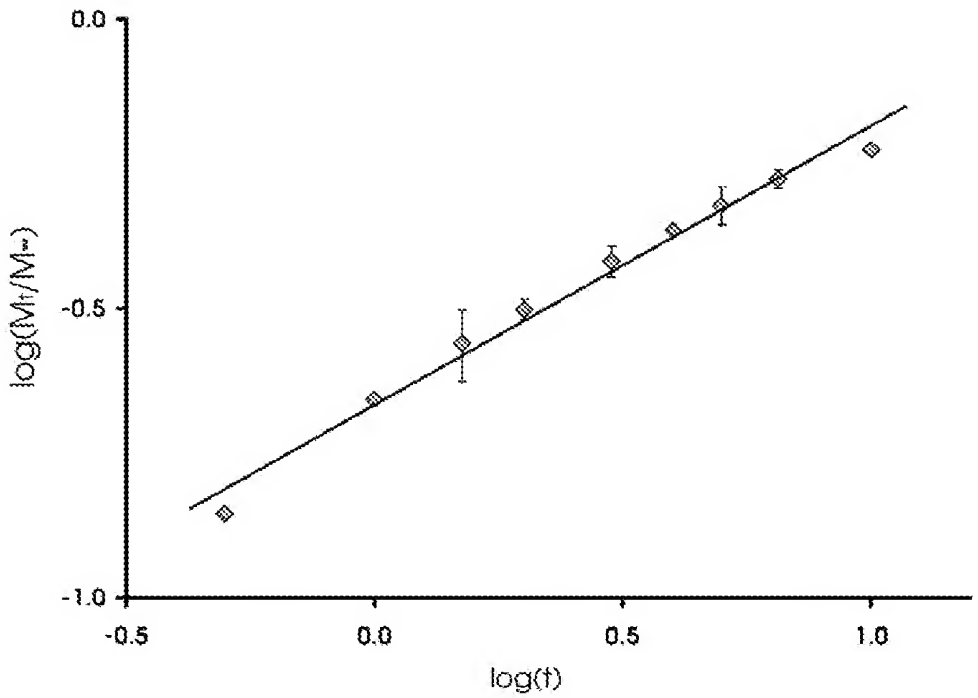

Fig. 6. The in situ release data of AG-1295-loaded nanospheres (170 nm) fitted by the diffusional release model by Ritger and Peppas [20,21] $M_t/M_\infty = kt^n$. A straight line is predicted for diffusion-controlled release presented as log(fractional release) versus log(time). The diffusional exponent derived from the graph slope ($n = 0.46 \pm 0.03$) is in agreement with a Fickian diffusion controlled release from a homogeneous population of matrix-type spheres.

temperature. The physical separation of the NS and the released drug partitioning into heptane considerably simplifies the task of developing a complementary drug assay, thus making this technique applicable with appropriate modifications to many water-insoluble agents. The results provided by the external sink method are, however, subject to distortion since they are affected by the rate of drug transition through the interface boundary, similarly to the standard release methods where the free drug is sampled from a medium separated from the drug carrier suspension. Although this evidently limits the method's use to comparative release studies, the simplicity and specific advantages of this technique make it a useful tool of investigation.

The two release techniques provided release profiles that differed in their pattern. A more pronounced initial and a flattened sustained phase distinguished the in situ release. 'Smoothed' release behavior typically observed with the external sink method was probably due to the initial release hindered by the phase separation. On the other hand, the higher capacity of the external sink medium appears to be the reason for the faster total release time-scale compared to the in situ release kinetics. Despite these differences, both methods revealed similar tendencies in drug release as a function of the NS formulation variables. The detailed discussion of the corresponding effects observed using the external sink method may be found elsewhere [3]. The external sink method was shown to be sufficiently sensitive for a qualitative characterization of the factors affecting the release behavior of the formulation, thus meeting the essential requirement applying to a comparative method.

The low acceptor capacity of water, a poor release medium for AG-1295, was purposely utilized for separation of the drug fractions differing in their

binding mode to the carrier NS. The deconvoluted external sink release profile of the loosely bound drug fraction was markedly different from the release pattern of encapsulated drug. Given their contrasting release behavior, the loosely bound drug appears to be a minor constituent of the total NS-associated AG-1295 in all studied formulations as implied by their release profiles. We hypothesized that the washable drug fraction, demonstrating higher release rate, is formed by the drug molecules adsorbed on the NS surface, while the slower kinetic component of the drug release should be attributed to AG-1295 entrapped in the PLA matrix. This hypothesis is supported by the strong similarity of the deconvoluted release profile with that of the drug adsorbed on blank NS. The adsorption of AG-1295 on PLA nanospheres was shown to be NS size-dependent: the steady state amounts of the surface-associated drug were 1.1 and 2.3 μg per mg PLA for 160 and 70 nm NS, respectively. The obtained inverse dependence of the adsorbed drug amount on the NS size might be predicted assuming spherical shape and same effective matrix density in the particles of both types. With these assumptions the total surface area of the NS available for the drug adsorption is directly proportional to the particle-forming polymer weight and inversely proportional to the nanosphere diameter. Interestingly, the adsorption rate was also shown to be dependent on the NS size (Fig. 4A). However, AG-1295 associated with the larger and smaller sized NS exhibited a similar release behavior, hence appearing to be mainly a function of the drug–NS binding mode. These results strongly suggest that the main part of AG-1295 in the nanoparticulate formulations is entrapped in the polymeric matrix, while surface-associated drug forms a variable, small fraction. The transition rate of AG-1295 between these pools is sufficiently slow to allow for their separation based on the different release rates of the drug in the respective states.

Previous studies demonstrated marked stability in vitro of NS composed of poly(D,L-lactide) with a molecular weight of 100 kDa [26,27]. Provided the short time period of AG-1295 in situ release observed in our study, polymeric matrix degradation obviously does not contribute significantly to the drug release. Alternatively, given the strong lipophilicity of the drug, the release of the encapsulated AG-1295 might be governed either by diffusion through the polymer matrix or by dissolution of the drug crystals depending on the drug state in the polymer. The drug solubility in PLA (75.0±4.9 mg/g) significantly exceeds the drug loading in the NS (1.5% W/W), indicating that the encapsulated AG-1295 apparently exists in the polymer as a solid solution or a homogeneous molecular dispersion; therefore its release is expected to occur predominantly by diffusion through the polymeric matrix.

While $t^{1/2}$ time dependence ($n=0.5$) is expected for the release by Fickian diffusion from a matrix-type sphere at early times, the first 60% of the fractional release are effectively described by a diffusional exponent of $n=0.43$ [20]. A diffusional exponent between 0.43 and 0.85 is encountered in release processes involving anomalous non-Fickian transport [20,21]. The n value of 0.46±0.3 observed in our case indicates that the release of AG-1295 is governed by Fickian diffusion. The results provided by this model should however be treated with caution, since the diffusional exponent was shown to be markedly biased by polydispersity of the sample [20,21]. Sinclair [22] and Peppas [28] also demonstrated that n is strongly influenced by the precision of the release data, often suggesting erroneous conclusions about the release mechanism despite 'satisfactory' data fitting by the model.

In contrast to the semi-empirical model, the ab initio diffusional model by Guy et al. [18] considers the basic formulation properties controlling the release kinetics. Assuming Fickian-type drug release, it provides an estimation of the drug diffusion coefficient indicative of the drug–polymer interaction. Polakovic et al. [29] implemented a similar model to analyze release of lidocaine from D,L-PLA-based NS. In accord with our results, the authors demonstrated a comparatively low diffusion coefficient of the drug, in the order of 10^{-16} cm^2/s, reflecting the strong hindrance effect of the polymer. It is noteworthy that our results show an alteration of the diffusion coefficient in the course of the drug release: the calculated diffusion coefficient equaled 4×10^{-16} and 2×10^{-16} cm^2/s for the early- and late-time release phase, respectively. Likewise, all nanoparticulate formulations in our study exhibited similar

change in their release kinetics. The discontinuous release pattern resulting in significant drug fraction retaining in the particulate carrier was reported previously for paclitaxel-loaded microspheres [30] and lidocaine-loaded nanospheres [31]. It is most likely that this phenomenon is associated with the different physical states of the polymer forming the outer layers and the core of the sphere. The formation of the nanosphere by the nanoprecipitation method presumably involves two distinct processes. During the first rapid phase the polymer migrating with the water-miscible solvent precipitates upon contact with water, thus forming the shell of a nanocapsule. In contrast, the slow phase of the nanosphere core formation is due to elimination of the retained organic solvent [32]. The final nanosphere, therefore, is composed of the outer polymer layers produced by the rapid precipitation, and the matrix core formed by the slow polymer desolvation. The contrasting diffusion rates of the drug entrapped in these two compartments, obviously differing in their molecular organization, results in the observed alteration of the formulation release behavior [33]. These findings, together with the results of the adsorbed drug study utilizing the external sink method, suggest that the release of tyrphostin AG-1295 from the PLA nanospheres represents a complex process. The burst phase, playing a minor role in the overall drug release from larger sized NS, but increasingly pronounced with a reduction in the NS size [3], is dominated by the rapid dissociation of the loosely surface-bound drug. The two subsequent phases of the drug release are governed by the diffusion of AG-1295 from the nanosphere matrix compartments showing different permeability characteristics in respect to the drug.

The biphasic in vivo elimination pattern of NS-associated AG-1295 observed in our previous study [4] was best described as a sum of two first-order kinetic processes. The slower component attributed to the NS strongly bound to the arterial tissue parallels the release kinetics of the drug provided fast in vivo degradation of unprotected AG-1295. Assuming the dominant role of diffusion in the drug release in vivo, an integral diffusion coefficient may be used to describe the release process. Despite the limited number of data points characterizing the release component of the elimination curve, the magnitude of the effective diffusion coefficient could be estimated. The calculated D value, in the order of 10^{-17} cm^2/s, reflects slower in vivo release kinetics of AG-1295 compared to our in vitro results. The dissociation of the drug, obstructed by an unstirred drug-saturated layer formed in proximity of the NS immobilized in the extracellular matrix, provides a plausible explanation for the lower D value observed in vivo.

5. Conclusions

In the present study we characterized the drug release properties of AG-1295-loaded PLA nanospheres prepared by nanoprecipitation. The two methods utilized for this purpose addressed the strong lipophilicity of the encapsulated compound and the release profile distortions inherent in conventionally used techniques. The methods were applied for a comparative study of drug release in nanoparticulate formulations, as well as for investigation of the release mechanism and drug–nanosphere interaction. The in vitro and in vivo drug release from the nanospheres apparently represents a complex process governed predominantly by diffusion.

Acknowledgements

This research was supported in part by the German-Israeli Foundation for Scientific Research and Development (GIF, # I-602-181.2). GG is a member of the David R. Bloom Center of Pharmacy at HUJI.

References

[1] S. Banai, Y. Wolf, G. Golomb, A. Pearle, J. Waltenberger, I. Fishbein, A. Schneider, A. Gazit, L. Perez, R. Huber, G. Lazarovichi, L. Rabinovich, A. Levitzki, S.D. Gertz, PDGF-receptor tyrosine kinase blocker AG1295 selectively attenuates smooth muscle cell growth in vitro and reduces neointimal formation after balloon angioplasty in swine, Circulation 97 (1998) 1960–1969.

[2] I. Fishbein, J. Waltenberger, S. Banai, L. Rabinovich, M. Chorny, A. Levitzki, A. Gazit, R. Huber, U. Mayr, S.D.

Gertz, G. Golomb, Local delivery of platelet-derived growth factor receptor-specific tyrphostin inhibits neointimal formation in rats, Arterioscler Thromb Vasc Biol 20 (2000) 667–676.

[3] M. Chorny, I. Fishbein, H.D. Danenberg, G. Golomb, Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics, J. Control. Release (2002) this issue.

[4] I. Fishbein, M. Chorny, S. Banai, A. Levitzki, H.D. Danenberg, J. Gao, X. Chen, E. Moerman, I. Gati, V. Goldwasser, G. Golomb, Formulation and delivery mode affect disposition and activity of tyrphostin-loaded nanoparticles in the rat carotid model, Arterioscler Thromb Vasc Biol 21 (2001) 1434–1439.

[5] R. Pecora, Dynamic light scattering measurement of nanometer particles in liquids, J. Nanoparticle Res. 2 (2000) 123–131.

[6] J.W. Kostanski, P.P. DeLuca, A novel in vitro release technique for peptide-containing biodegradable microspheres (article 4) AAPS PharmSciTech 1 (1) (2000) (http://www.pharmscitech.com).

[7] F.B. Landry, D.V. Bazile, G. Spenlehauer, M. Veillard, J. Kreuter, Release of the fluorescent marker Prodan® from poly(D,L-lactic acid) nanoparticles coated with albumin or polyvinyl alcohol in model digestive fluids (USP XXII), J. Control. Release 44 (1997) 227–236.

[8] M.Y. Levy, S. Benita, Drug release from submicronized o/w emulsion: a new in vitro kinetic evaluation model, Int. J. Pharm. 66 (1990) 29–37.

[9] B. Magenheim, M.Y. Levy, S. Benita, A new in vitro technique for the evaluation of drug release profile from colloidal carriers-ultrafiltration technique at low pressure, Int. J. Pharm. 94 (1993) 115–123.

[10] C. Washington, Evaluation of non-sink dialysis methods for the measurement of drug release from colloids: effects of drug partition, Int. J. Pharm. 56 (1989) 71–74.

[11] C. Washington, F. Koosha, Drug release from microparticulates; deconvolution of measurement errors, Int. J. Pharm. 59 (1990) 79–82.

[12] C. Washington, Drug release from microdisperse systems: a critical review, Int. J. Pharm. 58 (1990) 1–12.

[13] L. Illum, M.A. Khan, E. Mak, S.S. Davis, Evaluation of carrier capacity and release characteristics for poly(butyl 2-cyanoacrylate) nanoparticles, Int. J. Pharm. 30 (1986) 17–28.

[14] W. Lin, M.C. Garnett, S.S. Davis, E. Schacht, P. Ferruti, L. Illum, Preparation and characterisation of rose Bengal-loaded surface-modified albumin nanoparticles, J. Control. Release 71 (2001) 117–126.

[15] H.M. Redhead, S.S. Davis, L. Illum, Drug delivery in poly(lactide-co-glycolide) nanoparticles surface modified with poloxamer 407 and poloxamine 908: in vitro characterisation and in vivo evaluation, J. Control. Release 70 (2001) 353–363.

[16] G. Weber, F.J. Farris, Synthesis and spectral properties of a hydrophobic fluorescent probe: 6-propionyl-2-(dimethylamino)naphthalene, Biochemistry 18 (1979) 3075–3078.

[17] I. Fishbein, M. Chorny, L. Rabinovich, S. Banai, I. Gati, G. Golomb, Nanoparticulate delivery system of a tyrphostin for the treatment of restenosis, J. Control. Release 65 (2000) 221–229.

[18] R.H. Guy, J. Hadgraft, I.W. Kellaway, M.J. Taylor, Calculations of drug release rates from spherical particles, Int. J. Pharm. 11 (1982) 199–207.

[19] R.W. Baker, H.K. Lonsdale, Controlled release: mechanisms and rates, in: A.C. Tanquary, R.E. Lacey (Eds.), Controlled Release of Biologically Active Agents, Vol. 47, Plenum, New York, 1974, pp. 15–71.

[20] P.L. Ritger, N.A. Peppas, A simple equation for description of solute release. II. Fickian and anomalous release from swellable devices, J. Control. Release 5 (1987) 37–42.

[21] P.L. Ritger, N.A. Peppas, A simple equation for description of solute release. I. Fickian and non-Fickian release from non-swellable devices in the forms of slabs, spheres, cylinders or discs, J. Control. Release 5 (1987) 23–36.

[22] G.W. Sinclair, N.A. Peppas, Analysis of non-Fickian transport in polymers using simplified exponential expressions, J. Membrane Sci. 17 (1984) 329–331.

[23] C. Washington, Drug release from microparticulate systems, in: S. Benita (Ed.), Microencapsulation. Methods and Industrial Applications, Vol. 73, Marcel Dekker, New York, 1996, pp. 155–181.

[24] N.S. Magalhaes, H. Fessi, F. Puisieux, S. Benita, M. Seiller, An in vitro release kinetic examination and comparative evaluation between submicron emulsion and polylactic acid nanocapsules of clofibride, J. Microencap. 12 (1995) 195–205.

[25] G. Golomb, I. Fishbein, Tyrphostins, inhibitors of protein tyrosine kinase, in restenosis, Adv. Drug Deliv. Rev. 24 (1997) 53–62.

[26] A. Belbella, C. Vauthier, H. Fessi, J.P. Devissaguet, F. Puisieux, In vitro degradation of nanospheres from poly(D,L-lactides) of different molecular weights and polydispersities, Int. J. Pharm. 129 (1996) 95–102.

[27] D. Lemoine, C. Francois, F. Kedzierewicz, V. Preat, M. Hoffman, P. Maincent, Stability study of nanoparticles of poly(epsilon-caprolactone), poly(D,L-lactide) and poly(D,L-lactide-co-glycolide), Biomaterials 17 (1996) 2191–2197.

[28] N.A. Peppas, Release of bioactive agents from swellable polymers: theory and experiments, in: J.M. Anderson, S.W. Kim (Eds.), Recent Advances in Drug Delivery Systems, Plenum, New York, 1984, pp. 279–289.

[29] M. Polakovic, T. Görner, R. Gref, E. Dellacherie, Lidocaine loaded biodegradable nanospheres. II. Modelling of drug release, J. Control. Release 60 (1999) 169–177.

[30] R.T. Liggins, H.M. Burt, Paclitaxel loaded poly(L-lactic acid) microspheres: properties of microspheres made with low molecular weight polymers, Int. J. Pharm. 222 (2001) 19–33.

[31] T. Görner, R. Gref, D. Michenot, F. Sommer, M.N. Tran, E. Dellacherie, Lidocaine-loaded biodegradable nanospheres. I. Optimization of the drug incorporation into the polymer matrix, J. Control. Release 57 (1999) 259–268.

[32] P. Wehrle, B. Magenheim, S. Benita, The influence of process parameters on the PLA nanoparticle size distribution, evaluated by means of factorial design, Eur. J. Pharm. Biopharm. 41 (1995) 19–26.

[33] W.-I. Li, K.W. Anderson, P.P. DeLuca, Kinetic and thermodynamic modeling of the formation of polymeric microspheres using solvent extraction/evaporation method, J. Control. Release 37 (1995) 187–198.